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SFEC, a Sperm Flagellar Energy Carrier Protein

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Background

Sperm motility depends on a functional flagellum, which consist of several cytoskeletal components including the fibrous sheath. The fibrous sheath is a unique cytoskeletal component in the principal- piece segment of the mammalian sperm flagellum. The fibrous sheath surrounds the axoneme (a motile sliding apparatus) and outer dense fibers and defines the extent of the principal piece region of the sperm flagellum. It consists of two longitudinal columns connected by closely arrayed semicircular ribs that assemble from distal to proximal throughout spermiogenesis. A comprehensive review of the protein composition of the fibrous sheath was recently written (Eddy et al, *Microsc Res Tech.* 2003 May 1;61(1):103-15).

There are several functions for the fibrous sheath that have emerged to date. 1) The fibrous sheath functions as a protective girdle for the sperm axoneme while maintaining flagellar flexibility and affecting the plane of the flagellar beat. 2) The fibrous sheath, through its A kinase anchoring proteins AKAP 3 and AKAP4, serves as a scaffold for enzymes involved in signal transduction including protein kinase A, the Rho signaling pathway through rhoporrin and rhophilin, and presumably calcium signaling via CABYR [Naaby-Hansen et al 2002]. 3) The fibrous sheath anchors enzymes involved in the glycolytic pathway.

The concept that the fibrous sheath serves as a scaffold for glycolysis is based upon the light and electron microscopic localization of two enzymes of the glycolytic pathway, hexokinase 1 and glyceraldehyde 3 phosphate dehydrogenase to the ribs and longitudinal columns of the fibrous sheath. As described herein additional glycolytic pathway enzymes have now been associated with the fibrous sheath providing further evidence that the fibrous sheath serves as a scaffold for glycolysis. Furthermore applicants have now discovered a novel, sperm specific

fibrous sheath protein, that is believed to function as an adenine nucleotide translocase, and thus has been designated sperm flagellar energy carrier (SFEC).

Summary of Various Embodiments of the Invention

The present invention is directed to a sperm flagellar energy carrier protein (SFEC), antibodies specific for SFEC and nucleic acid sequences encoding said protein, as well as compositions comprising such compounds. SFEC is believed to be essential for sperm motility, and thus antagonists of SFEC activity are anticipated to have utility as contraceptive agents. Compositions comprising the amino acid, nucleic acid or antibodies of the present invention can also be used in accordance with the present invention as diagnostic indicators of fertility.

Brief Description of the Drawings

Fig. 1 is a photographic image of the isolated human fibrous sheath prepared by mechanical and chemical means as visualized using transmission electron microscopy.

Fig. 2 is an SDS-PAGE of isolated human fibrous sheath proteins.

Fig. 3 represents the peptides identified from microsequencing the C265 band and the human and mouse associated protein sequences.

Fig. 4 is a Northern blot of poly A RNA isolated from human spleen, thymus, prostate, testis ovary, small intestine, colon and leukocytes probed with ^{32}P labeled SFEC cDNA, demonstrating that SFEC is a testis specific protein.

Fig. 5 is a dot blot analysis of RNA from 76 different human tissues, again showing that SFEC is a testis specific protein.

Detailed Description of Embodiments

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as

used herein refers to a compound that is greater than 90% pure.

As used herein, the term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

A "polylinker" is a nucleic acid sequence that comprises a series of three or more closely spaced restriction endonuclease recognitions sequences.

"Operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, control sequences or promoters operably linked to a coding sequence are capable of effecting the expression of the coding sequence.

As used herein, "nucleic acid," "DNA," and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

The term "peptide" encompasses a sequence of 3 or more amino acids wherein the amino acids are naturally occurring or synthetic (non-naturally occurring) amino acids. Peptide mimetics include peptides having one or more of the following modifications:

1. peptides wherein one or more of the peptidyl --C(O)NR-- linkages (bonds) have been replaced by a non-peptidyl linkage such as a --CH_2-carbamate linkage (--CH_2OC(O)NR--), a phosphonate linkage, a -CH_2-sulfonamide (-CH_2--$S(O)_2$ NR--) linkage, a urea (--NHC(O)NH--) linkage, a --CH_2 -secondary amine linkage, or with an alkylated peptidyl linkage (--C(O)NR--) wherein R is C_1-C_4 alkyl;

2. peptides wherein the N-terminus is derivatized to a --NRR_1 group, to a -- NRC(O)R group, to a --NRC(O)OR group, to a --$NRS(O)_2R$ group, to a --NHC(O)NHR group where R and R_1 are hydrogen or C_1-C_4 alkyl with the proviso that R and R_1 are not both hydrogen;

3. peptides wherein the C terminus is derivatized to --C(O)R_2 where R_2 is selected from the group consisting of C_1-C_4 alkoxy, and --NR_3R_4 where R_3 and R_4 are independently selected from the group consisting of hydrogen and C_1-C_4 alkyl.

Naturally occurring amino acid residues in peptides are abbreviated as recommended by the IUPAC-IUB Biochemical Nomenclature Commission as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Norleucine is Nle; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; Glycine is Gly or G, and X is any amino acid. Other naturally occurring amino acids include, by way of example, 4-hydroxyproline, 5-hydroxylysine, and the like.

Synthetic or non-naturally occurring amino acids refer to amino acids which do not naturally occur *in vivo* but which, nevertheless, can be incorporated into the peptide structures described herein. The resulting "synthetic peptide" contains amino acids other than the 20 naturally occurring, genetically encoded amino acids at one, two, or more positions of the peptides. For instance, naphthylalanine can be substituted for trytophan to facilitate synthesis. Other synthetic amino acids that can be substituted into peptides include L-hydroxypropyl, L-3,4-dihydroxyphenylalanyl, alpha-amino acids such as L-alpha-hydroxylysyl and D-alpha-methylalanyl, L-alpha.-methylalanyl, beta.-amino acids, and isoquinolyl. D amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides. Other derivatives include replacement of the naturally occurring side chains of the 20 genetically encoded amino acids (or any L or D amino acid) with other side chains.

As used herein, the term "conservative amino acid substitution" is defined herein as an amino acid exchange within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:

Ala, Ser, Thr, Pro, Gly;

II. Polar, negatively charged residues and their amides:

Asp, Asn, Glu, Gln;

III. Polar, positively charged residues:

His, Arg, Lys;

IV. Large, aliphatic, nonpolar residues:

Met Leu, Ile, Val, Cys

V. Large, aromatic residues:

Phe, Tyr, Trp

As used herein, the term "SFEC polypeptide" and like terms refers to a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, and biologically active fragments thereof.

As used herein, the term "antibody" refers to a polyclonal or monoclonal antibody or a binding fragment thereof such as Fab, F(ab')2 and Fv fragments.

As used herein, the term "SFEC antibody" refers to an antibody that specifically binds to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4.

As used herein, the term "biologically active fragments" or "bioactive fragment" of an SFEC polypeptide encompasses natural or synthetic portions of the full-length protein that are capable of specific binding to their natural ligand.

The term "non-native promoter" as used herein refers to any promoter that has been operably linked to a coding sequence wherein the coding sequence and the promoter are not naturally associated (i.e. a recombinant promoter/coding sequence construct).

As used herein, a transgenic cell is any cell that comprises a nucleic acid sequence that has been introduced into the cell in a manner that allows expression of a gene encoded by the introduced nucleic acid sequence.

As used herein, the term "treating" includes alleviating the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms. For example, treating cancer includes preventing or slowing the growth and/or division of cancer cells as well as killing cancer cells.

Embodiments

Fertility requires sperm motility and consequently ATP production. Oxidative phosphorylation in mitochondria is the most efficient way to produce ATP but in the case of spermatozoa, the mitochondria are localized solely in the sperm mid piece and yet the flagella extends another 40 um or so beyond the base of the mid

piece. This raises the question of how ATP is generated and made available for the dynein-ATPases of the mitochondrion-free part of the flagellum (principal piece). Accordingly, it has been proposed that glycolysis along the flagellum provides a mechanism for localized ATP production in the principal piece and provides energy for the hyperactivated motility of sperm that allows them to penetrate the zona pellucida. In support of that hypothesis two enzymes of the glycolytic pathway, hexokinase 1 and glyceraldehyde 3 phosphate dehydrogenase have been localized to the fibrous sheath.

To further characterize the proteins that comprise the fibrous sheath, fibrous sheaths were isolated from human sperm using mechanical and biochemical dissection methods using techniques previously described (see Kim et al., Mol Hum Reprod. 1997 Apr;3(4):307-13). Electron microscopic observations of the dissected fraction revealed a highly purified preparation consisting exclusively of fibrous sheath ribs and longitudinal columns (see Fig. 1). The fibrous sheath proteins were extracted and one dimensional SDS-PAGE was conducted (see Kim et al., Mol Hum Reprod. 1997 Apr;3(4):307-13). 2-D gel analysis of the isolated fibrous sheath, using classical urea extraction methods, proved unsuccessful due to the insolubility of the fibrous sheath proteins in the Celis buffers employed in isoelectric focusing.

The results from one dimensional SDS-PAGE revealed that the fibrous sheath contains at least 17 distinct Coomassie staining protein bands. These bands were assigned a nomenclature of C253-C269, and each band was cored and microsequenced by tandem mass spectrometry. The results indicated that the isolated fibrous sheath preparation contained many proteins (see Table 1) that had been previously characterized as fibrous sheath components including roporrin, AKAP3, AKAP4, GST mu, and GAPDH-2. These findings confirmed the purity of the isolated fibrous sheath preparation. However, more significantly, microsequencing of isolated human fibrous sheath also revealed the presence of five glycolytic proteins, not previously reported to be associated with the fibrous sheath. These enzymes are aldolase A, sorbitol dehydrogenase, lactate dehydrogenase, triosphosphate iosmerase, pyruvate kinase. The addition of 5 new components to the 2 previously known glycolytic enzymes contained in the human fibrous sheath conclusively establishes glycolysis as a process occurring in the principal piece of the sperm flagellum, independent of ATP generation in the mitochondria. Glycolysis is an essential metabolic pathway that may proceed in the absence of oxygen to generate ATP.

Accordingly, these findings demonstrate that the fibrous sheath is a flagellar sub-compartment for the glycolytic pathway to generate ATP under anaerobic condition.

Preliminary bioinformatic analysis of the five glycolytic peptides that were obtained from the human fibrous sheath indicated that the glycolytic enzymes represent the somatic form each enzyme (see Table 2), with the exception of the testis specific form of lactate dehydrogenase, LDHC. Although testis isoforms of triose phosphate isomerase have been identified in human (Strausberg et al., Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16899-16903 (2002)), the peptides identified in the fibrous sheath represent the somatic form of TPI rather than the testis isoform. This indicates the fibrous sheath glycolytic machinery is comprised of two subsets of glycolytic enzymes: testis specific as well as somatic isoforms.

In addition several new uncharacterized hypothetical proteins were identified as components of the fibrous sheath. These include the hypothetical protein FLJ23338 from band C253, hypothetical protein R30953_1 from band C259 and hypothetical protein DKFZp434N1235 from band C265. The C265 band hypothetical protein DKFZp434N1235 has been cloned and sequenced and further studied by bio-informatic analysis. Genes were annotated by the Ensembl automatic analysis pipeline using either a GeneWise model form a human/vertebrate protein, a set of aligned human cDNAs followed by GenomeWise for ORF prediction for from Genscan exons supported by protein, cDNA and EST evidence. GeneWise models are further combined with available aligned cDNAs to annotate UTRs. Bio-informatic comparison of the band C265 protein with other known proteins revealed the highest homology (with a 69% identity and 79% similarity) to the amino acid sequence of adenine nucleotide translocase 1, ANT1, in human heart/skeletal muscle, and a 67% identity and 80% similarity to ANT3 of human liver. C265 protein also revealed a 67 % identity and 79 % similarity with a human fibroblast isoform (ANT2). The human SFEC protein is 315 amino acids in length, has a molecular weight of 35021.78 daltons, an isoelectric point of 10.4632, a charge of 24.5 and an average residue weight of 111.180. The functional domains of human SFEC are indicated in Table 3.

The nucleotide sequence of the human SFEC mRNA covers 1727 bp including an open reading frame that yields a protein of 315 amino acid residues. The gene structure of SFEC spans approximately 43.8 kb divided into 6 exons and 5 introns. The human SFEC gene was localized to chromosome 4q28.2, while murine

SFEC was localized to chromosome 3B. The other known human ADP/ATP carrier proteins in the same family such as heart/skeletal muscle isoformT1 (ANT 1) and liver isoformT2 (ANT 3) were localized to chromosome 4 q35.1 and chromosome X p22.33, respectively. Fibroblast isoform (ANT2) was localized to chromosome X q24. From this evidence indicating the presence of an uncharacterized unique gene the C265 protein is believed to be a novel member of the family of ADP/ATP Carrier Proteins, also known as the ADP/ATP Translocase, or alternatively, Adenine Nuleotide Translocator or ANT. Since the C265 protein was isolated from the fibrous sheath and because a role in signal transduction or glycolysis or both is likely, the novel protein has been designated as a sperm flagellar energy carrier protein or SFEC. At this time it is not yet apparent if SFEC functions as an ATP reserve [storage/sink] or as an ATP carrier which shuttles ATP to the axoneme.

It is known that testis specific isoforms [Hk1-sa, Hk1-sb and Hk1-sc] of hexokinase 1 are produced from a single somatic gene Hk1 [Mori et al 1993] by alternative splicing. In contrast the testis specific form of GAPDH, GAPDS, is encoded by a unique gene locus *Gapds* in mouse and *GAPDH2* in humans. Thus, of the two known glycolytic enzymes localized in the flagellum, testis specific isoforms exist, and these are generated by either alternative splicing or expression of unique genes. However, it is very interesting that the preliminary bioinformatic analysis of the peptides isolated from the human fibrous sheath indicates that they are all somatic isoforms and do not represent testis specific isoforms, although such forms have been described for triose phosphate isomerase [Strausberg *et al.*, 2002] and LDHC, the germ cell-specific member of the lactate dehydrogenase family [Millan et al., 1987; Strausberg *et al.*, 2002]. This supports the fibrous sheath as being comprised of testis specific and somatic members of the glycolytic enzyme families.

The nucleic acid sequences of human and mouse SFEC are shown as SEQ ID NO: 1 and SEQ ID NO: 3, respectively and the deduced human and mouse amino acid sequences are shown as SEQ ID NO: 2 and SEQ ID NO: 4, respectively. The human and mouse SFEC shared 83 % identity and 89% similarity of protein sequences.

In accordance with one embodiment of the present invention a purified polypeptide is provided comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4, or an amino acid sequence that differs from SEQ ID NO: 2 or SEQ ID NO: 4 by 1-5 conservative amino acid substitutions. In one embodiment the purified

polypeptide comprises an amino acid sequence that differs from SEQ ID NO: 2 by 20 or less conservative amino acid substitutions, and in another embodiment by 10 or less conservative amino acid substitutions. Alternatively, the polypeptide may comprise an amino acid sequence that differs from SEQ ID NO: 2 or SEQ ID NO: 4 by 1 to 5 alterations, wherein the alterations are independently selected from a single amino acid deletion, single amino acid insertion and conservative amino acid substitutions. In one embodiment the purified polypeptide comprises the amino acid sequence of SEQ ID NO: 2. The polypeptides of the present invention may include additional amino acid sequences to assist in the stabilization and/or purification of recombinantly produced polypeptides. These additional sequences may include intra- or inter-cellular targeting peptides or various peptide tags known to those skilled in the art. In one embodiment, the purified polypeptide comprises an amino acid of SEQ ID NO: 2 and a peptide tag, wherein the peptide tag is linked to SEQ ID NO: 2. In another embodiment, the purified polypeptide comprises an amino acid of SEQ ID NO: 4 and a peptide tag, wherein the peptide tag is linked to SEQ ID NO: 4. Suitable expression vectors for expressing such fusion proteins and suitable peptide tags are known to those skilled in the art and commercially available. In one embodiment the tag comprises a His tag.

The present invention also encompasses nucleic acid sequences that encode SFEC. In one embodiment a purified nucleic acid sequence is provided comprising the sequence of SEQ ID NO: 1, SEQ ID NO: 3 or a fragment of SEQ ID NO: 1 or SEQ ID NO: 3. The present invention also encompasses recombinant human SFEC gene constructs. In one embodiment, the recombinant gene construct comprises a non-native promoter operably linked to a nucleic acid sequence comprising SEQ ID NO: 1 or SEQ ID NO: 3. The non-native promoter is preferably a strong constitutive promoter that enables expression of the gene construct in a predetermined host cell. These recombinant gene constructs can be introduced into host cells to produce transgenic cell lines that synthesize the SFEC gene products. Host cells can be selected from a wide variety of eukaryotic and prokaryotic organisms, and two preferred host cells are *E. coli* and yeast cells.

In accordance with one embodiment, a nucleic acid sequence comprising SEQ ID NO: 1 or SEQ ID NO: 3 is inserted into a eukaryotic or prokaryotic expression vector in a manner that operably links the gene sequence to the appropriate regulatory sequences, and SFEC is expressed in the eukaryotic or

prokaryotic host cell. In one embodiment the gene construct comprises the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 operably linked to a eukaryotic promoter. Suitable eukaryotic host cells and vectors are known to those skilled in the art. The baculovirus system is also suitable for producing transgenic cells and synthesizing the SFEC genes of the present invention. One aspect of the present invention is directed to transgenic cell lines that express human SFEC and fragments of the human SFEC coding sequence.

In one embodiment the introduced nucleic acid is sufficiently stable in the transgenic cell (i.e. incorporated into the cell's genome, or present in a high copy plasmid) to be passed on to progeny cells. The cells can be propagated *in vitro* using standard cell culture procedure, or in an alternative embodiment, the host cells are eukaryotic cells and are propagated as part of a non-human animal, including for example, a non-human transgenic animal. In one embodiment the transgenic cell is a human cell propagated *in vitro* and comprises the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3.

The present invention also encompasses a method for producing human and mouse SFEC. The method comprises the steps of introducing a nucleic acid sequence comprising a sequence that encodes the human or mouse SFEC into a host cell, and culturing the host cell under conditions that allow for expression of the introduced SFEC gene. In one embodiment the promoter is a conditional or inducible promoter, alternatively the promoter may be a tissue specific or temporal restricted promoter (i.e. operably linked genes are only expressed in a specific tissue or at a specific time). The synthesized SFEC can be purified using standard techniques and used in high throughput screens to identify inhibitors of SFEC activity. Alternatively, in one embodiment the recombinantly produced SFEC polypeptides, or fragments thereof are used to generate antibodies against the human or mouse SFEC. The recombinantly produced SFEC proteins can also be used to obtain crystal structures. Such structures would allow for crystallography analysis that would lead to the design of specific drugs to inhibit SFEC function.

Consistent with SFEC's sequence similarity to other known ADP/ATP carrier proteins and its testis specific expression, the new fibrous sheath protein, SFEC is anticipated to have a function related to the diffusion of ATP (produced by glycolysis) through the principal piece of the flagellum. SFEC may function either as an energy carrier protein for sperm motility or alternatively, as a reservoir of ATP or

ADP. Accordingly, this protein represents a target for a small molecule inhibitor that is anticipated to have a contraceptive effect. Such an inhibitor might be effective as either a male contraceptive or an intravaginal spermicidal product.

In accordance with one embodiment of the present invention a method is provided for isolating agents that inhibit SFEC activity and thus serve as contraceptive agents. More particularly, in one embodiment agents will be screened for their ability to interfere with SFEC's ability to bind ADP and/or ADP. Small molecules that are capable of penetrating the sperm plasma membrane will be highly desirable. In addition the small molecule inhibitors should not be toxic to somatic cells. Isolated SFEC inhibitors will be used in accordance with the present invention either alone or in conjunction with other contraceptive agents to prevent unintended pregnancies.

In accordance with another embodiment of the present invention an antigenic composition is provided comprising a purified the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 4 or an antigenic fragment thereof. The composition can be combined with a pharmaceutically acceptable carrier or adjuvant and administered to a mammalian species to induce an immune response. Such antigenic compositions have utility for raising antibodies against the SFEC protein for use in diagnostic purposes, or in one embodiment for use in contraceptive vaccine formulations. The vaccines of the invention may be multivalent or univalent. Multivalent vaccines are made from recombinant viruses/vectors that direct the expression of more than one antigen.

Suitable preparations of antigenic compositions include injectables, either as liquid solutions or suspensions; solid forms suitable for solution (or suspension) in liquid prior to injection, may also be prepared. The preparation may also be emulsified, or the polypeptides encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the antigenic composition may also include minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Examples of adjuvants which may be effective, include, but are not limited to: mineral gels, *e.g.*, aluminum hydroxide, surface active substances such as

lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; alum, and MDP; N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine, aluminum hydroxide;.

The polypeptides may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids, such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with free carboxyl groups may also be derived from inorganic bases, such as, for example, sodium potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

The present invention also encompasses antagonists and agonists, including compounds or nucleotide constructs that inhibit expression or the activity of human SFEC (i.e. transcription factor inhibitors, antisense, interference RNA and ribozyme molecules, or gene or regulatory sequence replacement constructs) as well as antibodies that interfere with the activity of SFEC. Antagonists of SFEC activity can be used as contraceptive agents. In accordance with one embodiment a method for identifying antagonists of SFEC activity is provided. The method comprises the steps of contacting an SFEC protein, in the presence and absence of a potential SFEC antagonist, with ATP or ADP or other adenosine derivative and identifying antagonists of SFEC activity based on the ability of said potential SFEC antagonist to decrease binding of ATP or ADP or other adenosine derivative to SFEC. In one embodiment the SFEC protein comprises an amino acid sequence of SEQ ID NO: 2. The present invention also encompasses a method of providing contraception to mammalian species, said method comprising the steps of contacting mammalian sperm cells with a composition comprising an inhibitor of SFEC activity.

In accordance with one embodiment of the present invention an antibody is provided that specifically binds to the human and/or mouse SFEC polypeptide (i.e. SEQ ID NO: 2 or 4). In accordance with one embodiment an antibody is provided that specifically binds to the polypeptide of SEQ ID NO: 2. Antibodies generated in accordance with the present invention may include, but are not limited to, polyclonal, monoclonal, chimeric (i.e "humanized" antibodies), single

chain (recombinant), Fab fragments, and fragments produced by a Fab expression library. These antibodies can be used as diagnostic agents for the diagnosis of conditions or diseases characterized by in appropriate expression or overexpression of SFEC (including neoplastic disease), or in assays to monitor the effectiveness of an SFEC agonist, antagonist or inhibitor. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule. In addition, the antibodies can be formulated with standard carriers and optionally labeled to prepare therapeutic or diagnostic compositions.

Antibodies raised against SFEC can be generated using standard techniques, and include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and Fab expression libraries. The antibodies generated can be formulated with standard carriers and optionally labeled to prepare therapeutic or diagnostic compositions. In one embodiment, a composition is provided comprising a SFEC specific antibody and a pharmaceutically acceptable carrier. In one embodiment the composition further comprises a surfactant, adjuvant, excipient or stabilizer. In general, water, saline, aqueous dextrose, and related sugar solution, and glycols such as, propylene glycol or polyethylene glycol, are the liquid carriers, particularly for injectable solutions.

Various procedures known in the art may be used for the production of polyclonal antibodies to SFEC or derivatives or analogs thereof. For the production of antibody, various host animals, including but not limited to rabbits, mice, rats, etc can be immunized by injection with the polypeptide of SEQ ID NO: 2, SEQ ID NO: 4, or a synthetic version, or derivative (*e.g.*, fragment) thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward the sequence of SEQ ID NO: 2, SEQ ID NO: 4, or fragment thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and

Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In one embodiment, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for egg surface proteins together with genes from a human antibody molecule of appropriate biological activity can be used; such "humanized" antibodies are within the scope of this invention.

According to the present invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce SFEC single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for SFEC, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of SFEC, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc. The antibodies generated against SFEC antigens can also be used as contraceptive or sterilization agents (i.e. passive immunotherapy), or for use in

diagnostic immunoassays or the generation of antiidiotypic antibodies. For example, in one embodiment SFEC antibodies are isolated (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in diagnostic immunoassays, or the antibodies may be used to monitor treatment and/or disease progression. Any immunoassay system known in the art, such as those listed *supra*, may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme-linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays.

Another embodiment of the present invention is directed to small molecule inhibitors of SFEC and their use to decrease the motility of mammalian sperm and thus serve as a contraceptive agent. In one embodiment a method of contraception is provided wherein said method comprises the steps of inhibiting the activity of SFEC. Alternatively, the SFEC inhibitory composition may comprise an antisense or interference RNA that prevents or disrupts the expression or activity of SFEC in mammalian sperm cells. In accordance with one embodiment the fertility inhibiting composition comprises one or more active agents selected from the group consisting of small molecule inhibitors, antibodies, antisense RNA and interference nucleic acid sequences.

Interference RNA in mammalian systems requires the presence of short interfering RNA (siRNA), which consists of 19-22nt double-stranded RNA molecules, or shRNA, which consists of 19-29nt palindromic sequences connected by loop sequences. Down regulation of gene expression is achieved in a sequence-specific manner by pairing between homologous siRNA and target RNA. A system for the stable expression of siRNA or shRNA was utilized to generate transgenic animals (Hasuwa et al. FEBS Lett 532, 227-30 (2002), Rubinson et al. Nat Genet 33, 401-6 (2003) and Carmell et al. Nat Struct Biol 10, 91-2 (2003)) and can be used in accordance with the present invention to produce animals whose fertility can be regulated. A conditional interference RNA-based transgenic system would provide the additional benefit of being able to control the level of gene expression at any given stage during the life of the animal. Such a regulatable system would have value in livestock and domesticated animals.

Claims

1. An antibody that specifically binds to an SFEC polypeptide.

2. The antibody of claim 1 wherein the antibody specifically binds to the polypeptide of SEQ ID NO: 2.

3. The antibody of claim 1 wherein said antibody is a monoclonal antibody.

4. A composition comprising the antibody of claim 1 and a pharmaceutically acceptable carrier.

5. An antigenic composition comprising an amino acid sequence comprising SEQ ID NO: 2 or an antigenic fragment thereof and a pharmaceutically acceptable carrier.

6. The composition of claim 5 further comprising an adjuvant.

SFEC, a Sperm Flagellar Energy Carrier Protein

Abstract of the Disclosure

The present invention is directed to a sperm flagellar energy carrier, SFEC, antibodies specific for the SFEC and the use of the SFEC protein to identify antagonists of SFEC activity. SFEC is believed to be essential for sperm motility, and thus antagonists of SFEC activity are anticipated to have utility as contraceptive agents.

SEQUENCE LISTING

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Herr, John
Hao, Zhonglin

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    290                 295                 300

Ile Lys Glu Phe Leu Asn Ile Asp Val Gly Gly Ser Ser Ser Gly Asp
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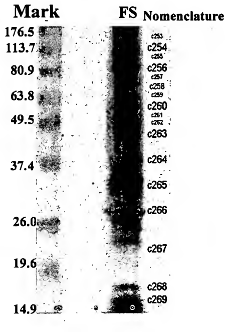
```

Fig 1. Isolation of human Fibrous Sheath

Human sperm fibrous sheath was isolated using mechanical and chemical sperm dissection methods as previously described (Kim *et al.*, 1997). The purity of isolation was confirmed by a transmission electron microscopy.

Fig 2. SDS-PAGE of human fibrous sheath proteins



SDS-PAGE of human fibrous sheath protein (**FSP**) revealed at least 17 Coomassie stained bands. A nomenclature for these were established (Table 1)

Fig

3. Microsequencing of Fibrous Sheath Proteins

Each band of fibrous sheath protein was microsequenced by mass spectrometry. The sequence result was summarized in table 1. The band C265 was identified as an unknown protein (DKFZp434N1235). Peptides microsequenced from the C265 band are indicated by bold underlines.

Microsequencing of C265 by Mass Spectrometry

mhrepakkka ekr**lfdassf gkdllaggva aavsk**tavap iervklllqv qasskqispe
arykgmvdcl vripreqgff sfwrgnlanv iryfptqaln fafkdkykql fmsgvnkekq
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ydkikeffhi diggr

Mouse Orthologous of SFEC

msnesskkqs skkalfdpvs fskdllaggv aaavskttva piervklllq vqasskqisp
earykgmldc lvripreqgf lsywrgnlan viryfptqal nfafkdkyke lfmsgvnkek
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sypfdtvrrr mmmqsgesdr qykgtidcfl kiyrhegvpa ffrgafsnil rgtggalvlv
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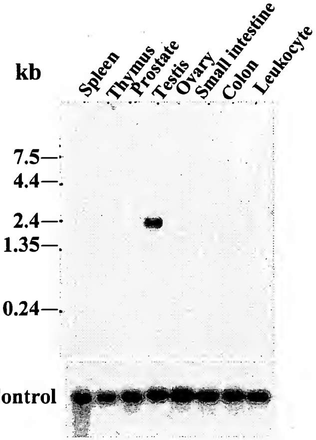
Fig 4

6. SFEC is a testis specific Protein

Northern and Dot blot analyses demonstrated that the SFEC is a testis specific protein

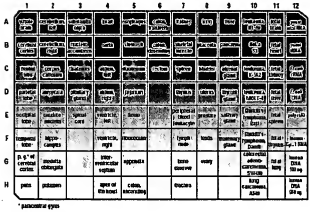
Northern Analysis



Northern blot analysis of human multi-tissue poly A^+ RNA; SFEC cDNA corresponding to full length of ORF was radiolabeled with ^{32}P and hybridized to 2μg poly$(A)^+$ mRNAs revealing 2.4-kb message only in testicular RNA. Size of molecular weight markers is indicated at left. Human Northern blot used for SFEC cDNA was stripped and hybridized with ^{32}P-labeled cDNA of β–actin as a positive control.

Fig 5

Dot blot Analysis



Human multiple tissue expression (MTE) array containing normalized loadings of poly A$^+$ RNA from 76 different human tissues (see diagram at right) probed with ^{32}P-labeled human SFEC cDNA demonstrating its testis specific expression. *E.Coli* DNA was also hybridized.

GenBank Submission (see attached)

Genbank has provided GenBank accession numbers

Human: AY550240
Mouse: AY550241

Attorney Docket No. 00973-01

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

22427 U.S. PTO 031704

13441 U.S. PTO 60/554085 031704

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

Certificate Under 37 CFR 1.10
Date of Deposit: March 17, 2004
I hereby certify that this correspondence is being deposited with the United States Postal Service as "Express Mail" service under 37 CFR 1.10 on the date indicated above addressed to Mail Stop Provisional Application, Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450

Sue Ann Carr
Express Mail No. ER 861 428 174 US

Address to: Mail Stop Provisional Application
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450
(703) 308-4357

This is a request for filing a Provisional Application for Patent under 37 CFR 1.53(c)

Inventor(s) and Residence(s) (city and either state or foreign country):

Last Name	First Name	Middle Initial	City	State or Country
Hao	Zhonglin		Charlottesville	Virginia
Herr	John	C.	Charlottesville	Virginia

Title: **SFEC, A SPERM FLAGELLAR ENERGY CARRIER PROTEIN**

17	Sheets of specification.
5	Sheets of sequence listing.
4	Sheets of tables.
4	Sheets of drawings.

University of Virginia Patent Foundation claims small entity status as a nonprofit organization (37 CFR §§1.27(a)(3) and (c)). The Commissioner is hereby authorized to charge the Small Entity Fee of **$80** to **Deposit Account No. 50-0423.**

Please direct all communication relating to this application to:
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Customer No. 34444
Telephone: (434) 243-6103
Fax: (434) 924-2493

This invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. The government has certain rights in the invention.

YES ☒ NO ☐ Grant No. NIH TW 00654

Dated: March 17, 2004

Respectfully submitted,

By: John P. Breen (Reg. No. 38,833)